activity when kept frozen at −20°C (Fraction 6, Table 1).

The purification has usually been run with about 80 g of lyophilized mucosa extracts as starting material in each preparation. It has regularly resulted in an enzyme preparation having about 500 times higher specific activity than that of the acid extract. The overall recovery in the purification procedure has been about 5 %, equivalent to about 100 μg purified enzyme protein per gram of lyophilized mucosa extract.

This investigation was supported by grants from the *Swedish Medical Research Council* (Project No. 13X-17), and *Albert Påhlssons stiftelse*.

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Received May 14, 1971.

Preparation of a Highly Purified Glycyl-L-leucine Dipeptidase from Pig Intestinal Mucosa

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The final digestion of proteins in the intestine involves several separate dipeptide splitting enzymes. Our knowledge of these enzymes has so far been based on their behaviour in crude extracts or in partly purified preparations. A more reliable information about their enzymatic properties and molecular constitution requires, however, their purification. In the following we wish to report a method for purification of a glycyl-L-leucine dipeptidase (glycyl-L-leucine hydrolase, EC 3.4.3.2) from pig intestinal mucosa.

Glycyl-L-leucine dipeptidase activity was determined under optimal pH-conditions at 25°C according to the method of Josefsson and Lindberg,[1] using glycyl-L-leucine (Sigma, Lot. No. 127 B−1460) as substrate. No metal ions were added. One unit (U) of enzyme activity is defined as the activity hydrolyzing 1 μmol/min of glycyl-L-leucine at 25°C. The absorbance at 280 nm was used as a measure of protein concentration. All purification procedures were performed in the cold room. Besides at the $(NH_4)_2SO_4$ precipitations, all buffers contained 2-mercaptoethanol (4 mM) and glycerol (12.5 % w/v) in order to stabilize the enzyme activity.

Fresh pieces (3 m taken 2 m distal from pylorus) of small intestines of adult pigs were used as source of enzyme. After being squeezed to remove intestinal content they were cut open and divided into 5 cm pieces. Without further disintegration the pieces were subsequently extracted with stirring for 1 h in precooled distilled water (1 l per 3 m intestine). After centrifugation the supernatant was lyophilized. The lyophilized, brown-yellow powder (90 g/30 m intestine) was suspended in 0.05 M phosphate (pH 7.5). Insoluble material was removed by centrifugation. The opalescent supernatant (Fraction 1, Table 1) was fractionated with $(NH_4)_2SO_4$. The fraction, precipitating between 40 and 65 % saturation of

Table 1. Purification of a glycyl-L-leucine dipeptidase from pig intestinal mucosa.

Fraction	Vol. (ml)	A_{280}	Total activity (U)	Yield (%)	Purification factor
1. Supernatant	897	69.0	60 500	100	1
2. Dialyzed $(NH_4)_2SO_4$ precipitate	99	52.9	32 100	53	6.2
3. Pooled active fractions of DEAE-cellulose effluent	143	2.71	32 300	53	85
4. Pooled active fractions of DEAE-Sephadex effluent	129	0.381	25 500	42	531
5. Pooled active fractions of hydroxylapatite effluent	38	0.295	14 100	23	1 290
6. Pooled active fractions of Sephadex G-100 effluent	38.5	0.063	4 900	8.1	2 060

Figures are given for 90 g lyophilized intestinal extract.

$(NH_4)_2SO_4$ (pH 7.5), was dissolved in 0.05 M phosphate (pH 3.0). The pH of the solution was adjusted to 6.2 by 0.1 M NaOH and then again fractionated with $(NH_4)_2SO_4$. The fraction, precipitating between 20 and 35 % saturation of $(NH_4)_2SO_4$, was dissolved in 0.02 M Tris-HCl (pH 7.3) and dialyzed against the same buffer for 18 h. A small insoluble residue, formed during dialysis, was centrifuged off and the almost clear supernatant was adjusted to pH 7.3 (Fraction 2, Table 1).

The solution was applied to a 2.8 cm × 27 cm column of DEAE-cellulose (Whatman, DE 32) prepared and equilibrated in 0.02 M Tris-HCl (pH 7.3). The column was eluted with a linear increasing NaCl-gradient in the same buffer. The glycyl-L-leucine dipeptidase activity was eluted as a single peak and the effluent containing more than 100 U/ml of activity was collected (Fraction 3, Table 1). The enzyme solution was diluted with 0.001 M Tris-HCl (pH 7.3) to obtain the same conductivity as that of 0.05 M Tris-HCl (pH 7.3), made 0.04 M in respect to NaCl. The solution was then applied to a 2.8 cm × 30 cm column of DEAE-Sephadex A-50 (Pharmacia), prepared and equilibrated in the second buffer. The column was eluted with a linear increasing NaCl-gradient in the buffer. The glycyl-L-leucine dipeptidase activity was again eluted as a single peak, and the effluent containing more than 75 U/ml of activity were collected (Fraction 4, Table 1). The enzyme solution was subsequently applied to a 2.8 cm × 17 cm column of hydroxylapatite (Bio-Gel HTP), prepared and equilibrated in 0.025 M phosphate (pH 7.0), made 0.2 M in respect to NaCl. The column was eluted with stepwise increasing concentrations of phosphate. The glycyl-L-leucine dipeptidase activity was eluted as a single, narrow peak at 0.07 M phosphate. The effluent containing more than 90 U/ml of activity was collected (Fraction 5, Table 1). The enzyme solution was concentrated 18 times by means of ultrafiltration (Amicon, Diaflo filter PM 10) and applied to a column of Sephadex G-100 (Pharmacia). The column, prepared and equilibrated in 0.025 M phosphate (pH 7.0), containing 0.2 M NaCl, was eluted with the buffer and the effluent was assayed for protein and for enzyme activity (Fig. 1).

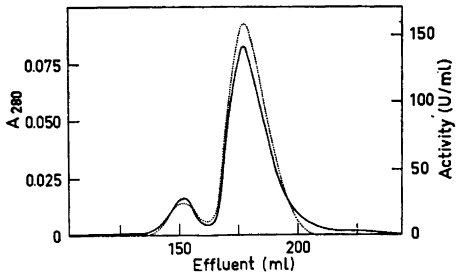

Fig. 1. Chromatography of glycyl-L-leucine dipeptidase on Sephadex G-100. 1 ml of concentrated active fractions from a hydroxylapatite column was applied to a 2.5 cm × 91 cm column equilibrated and eluted with 0.025 M phosphate (pH 7.0), containing 0.2 M NaCl. Flow rate 10 ml/h. A_{280} (———); enzyme activity (· · ·).

The activity was eluted as two separated peaks, of which the minor peak corresponded to less than 5 % of the total yield of enzyme activity.

The major peak of activity was collected, concentrated by ultrafiltration as above, and analyzed for homogeneity in polyacrylamide gel electrophoresis.[2] In order to localize the glycyl-L-leucine dipeptidase activity in the polyacrylamide gel and relate it to the protein, the gel was cut longitudinally into two halves. One half was stained with Coomassie Blue and the other was cut in 1 mm thick slices, which were separately extracted in 0.02 M Tris-HCl (pH 7.3), and assayed for enzyme activity. The result of a typical experiment is shown in Fig. 2. Repeated gel electrophoresis of the major band after concentration showed the same distribution of protein into three bands. This result indicates a transformation between different forms of the enzyme occurring during the experiment, a phenomenon known from other polyacrylamide gel electrophoresis experiments.[3]

Fig. 2. Polyacrylamide gel electrophoresis (pH 9.3) of glycyl-L-leucine dipeptidase. 100 μg of the major peak of a Sephadex G-100 chromatography were applied. Most activity was found in relation to the strongest band but the weaker bands also corresponded to traces of activity. The gel was stained with 0.04 % Coomassie Brilliant Blue in 10 % trichloroacetic acid. 2.5 mA was applied for 90 min.

Each purification usually starts with about 90 g of lyophilized mucosa extract and gives about 2 mg of the purified glycyl-L-leucine dipeptidase (major peak of Sephadex G-100 effluent, Fraction 6, Table 1).

This investigation was supported by grants from the *Swedish Medical Research Council* (Project No. 13X-17), and *Albert Påhlssons stiftelse.*

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Received May 14, 1971.

Conformational Spectroscopic Studies of *trans*-1,2-Bromoiodocyclohexane

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We have recently reported new infrared and Raman spectral data for various monohalo [1] and *trans*-1,2-dihalocyclohexanes.[2-4] By these methods we have studied the conformational equilibrium in the liquid, in various solutions and in the crystalline states. A detailed study of the infrared and Raman spectra of these molecules has revealed a remarkable similarity between their spectra.

Whereas the *e*-conformer is the more stable in all the halocyclohexanes,[1] the *aa* conformer becomes increasingly stabilized relative to *ee* with heavier halogens in the *trans*-1,2-dihalocyclohexanes.[2-4] Thus, the dichloro derivative [2] crystallizes in *ee*, and the dibromo [2] and chloroiodo derivatives [4] crystallize in *aa*, whereas the intermediate molecule bromochlorocyclohexane is present as *ee* in the low temperature and as *aa* in the high pressure solid.[2,3]

These results have encouraged us to synthesize *trans*-1,2-bromoiodocyclohexane (BIC). However, it turned out that this molecule was quite unstable at room temperature and rapidly turned red because of free halogen. Therefore our spectral data, reported in the present communication, are not as complete as those obtained for the previous molecules.